Kód předmětu: Bi8980

MASARYKOVA UNIVERZITA

Protein expression and purification

- IX. **Protein quality**

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Tento projekt je spolufinancován Evropským sociálním fondem a státním rozpočtem České republiky.

EVROPSKÁ UNIE

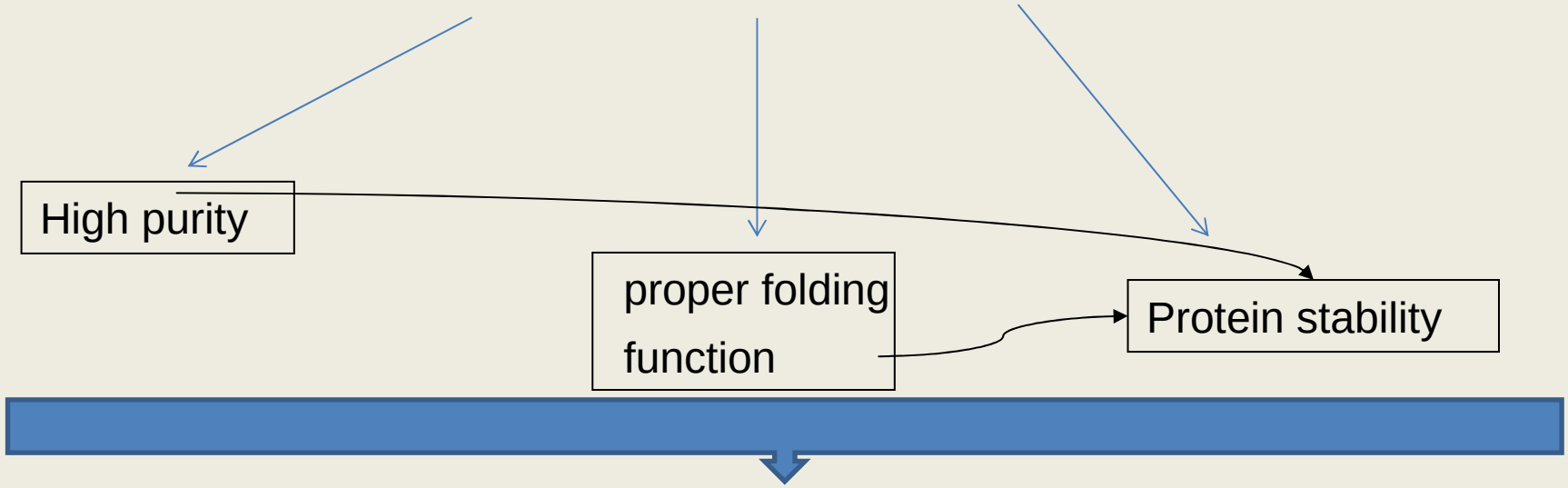
MINISTERSTVO ŠKOLSTVÍ, MLÁDEŽE A TĚLOVÝCHOVY

OP Vzdělávání pro konkurenceschopnost

INVESTICE DO ROZVOJE VZDĚLÁVÁNÍ

Source of protein → Extraction → Separation → Characterization

Quality parameters of produced protein



High quality of produced protein

3D structure determination
Detail function analysis

How to determine the protein quality parameters?

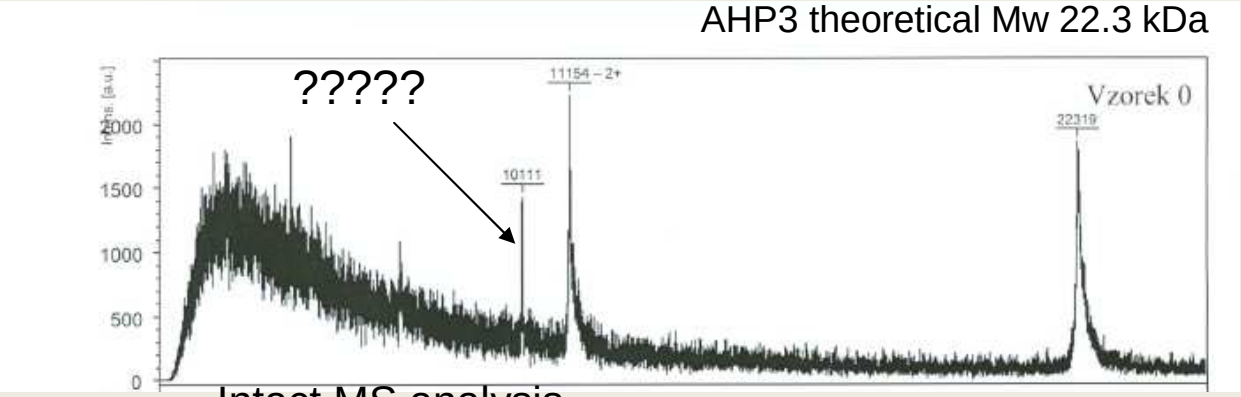
PROTEIN PURITY

= protein is homogenous in its native state

How to determine protein purity?

- **electrophoresis (SDS, native)**
- **gel filtration**
- mass spectrometry (MS)
- **dynamic light scattering**

AHP3 theoretical Mw 22.3 kDa



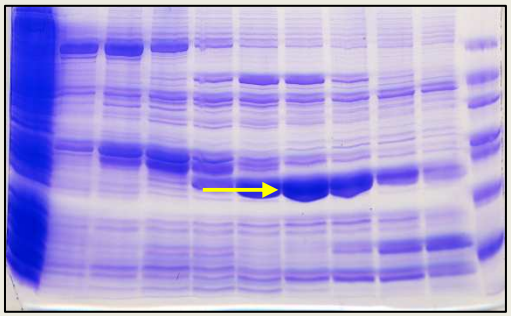
Intact MS analysis

PROTEIN PURITY

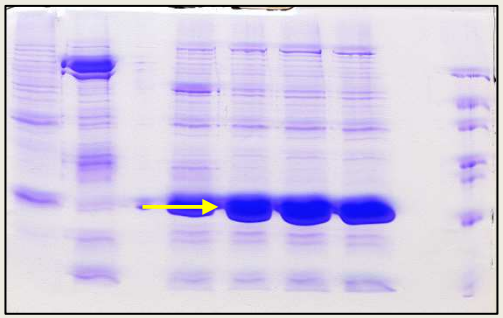
SDS-PAGE

- For a pure protein, only one band is expected (5, 10, 20 μg of protein/lane of the gel).
- Mw determination (information about protein subunits)
- Protein purity is evaluated in SDS-PAGE using various software (e.g. Quantity One from Bio-RAD).
- Definition of protein purity: a quantity of particular band as measured by its intensity, expressed as a percentage of the total intensity of all bands in the lane of the gel

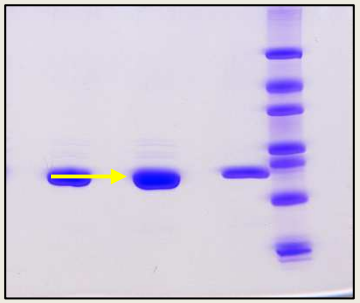
28% purity



80% purity



95% purity

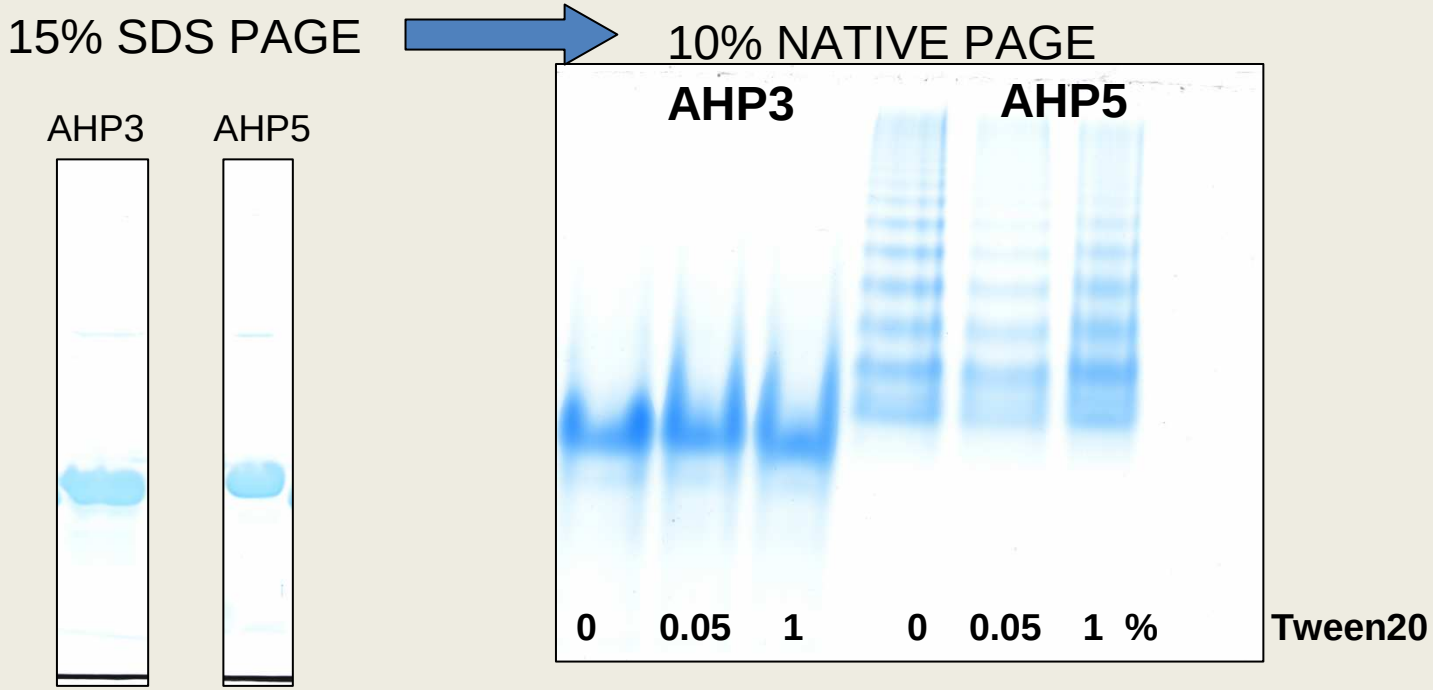


PROTEIN PURITY

- **NATIVE PAGE**
- **PFO NATIVE PAGE**

- The method was developed for analyzing native multimeric structure of many proteins.

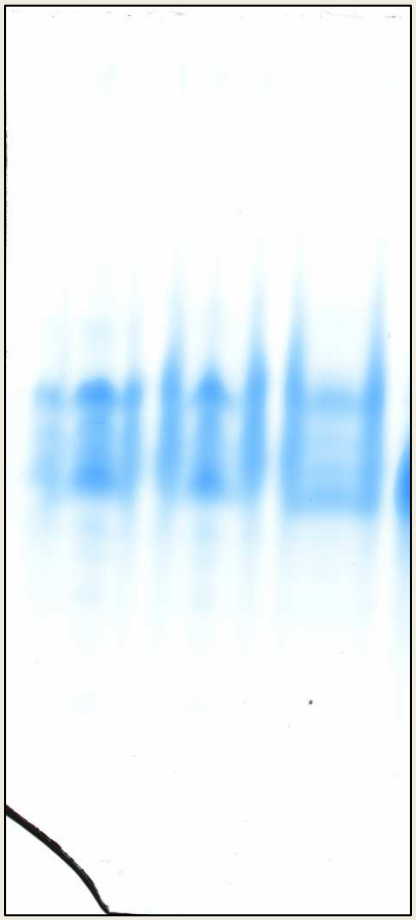
- PFO (perfluoro-octanoic acid) protects interaction within protein oligomers and enables the determination of molecular mass for multimeric proteins.



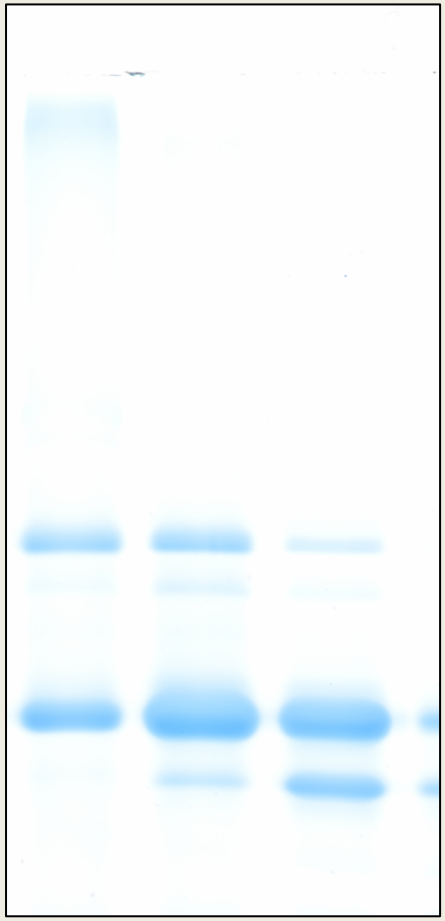
NATIVE PAGE x PFO NATIVE PAGE

AHP2

10% NATIVE PAGE



15% PFO NATIVE PAGE



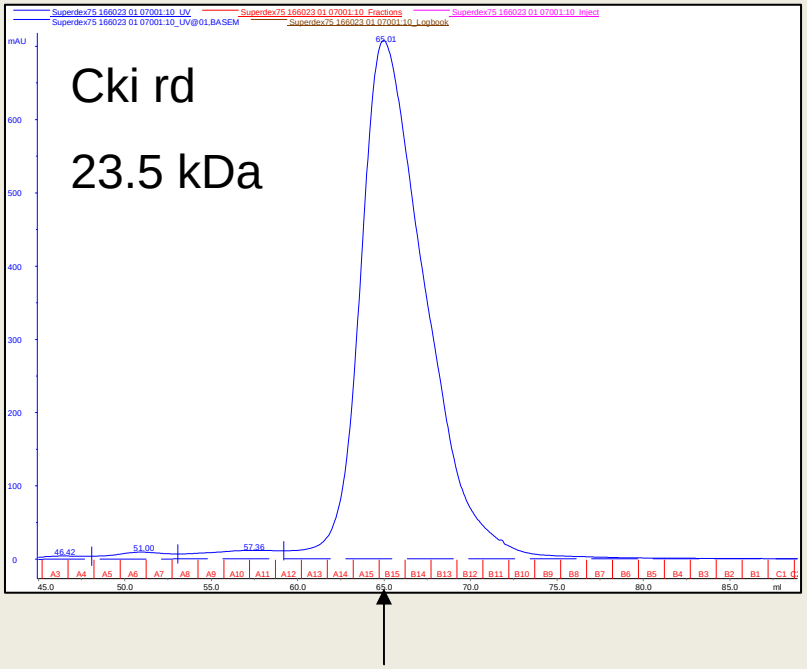
Gel filtration

- **Gel filtration chromatography separates proteins on the basis of size.**

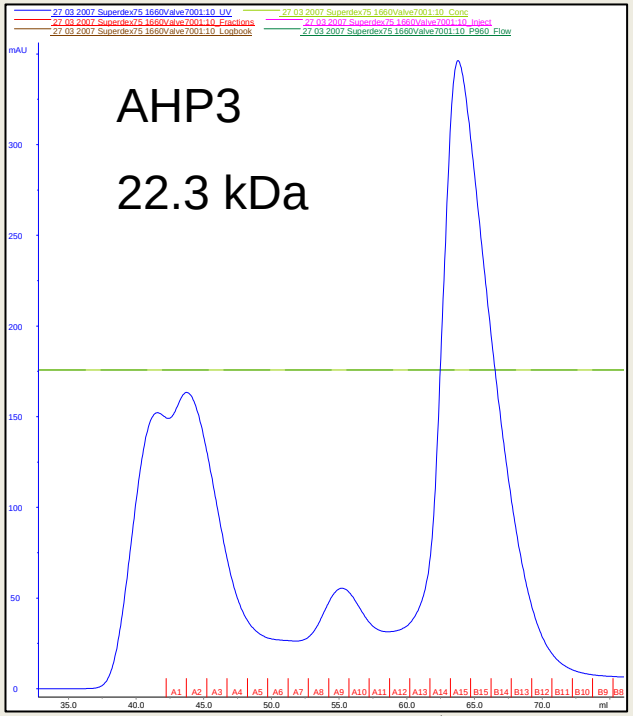
- **Molecules move through a bed of porous beads. Smaller molecules diffuse further into the pores of the beads and therefore move through the bed more slowly, while larger molecules enter less or not at all and thus move through the bed more quickly.**

- **Both molecular weight and three-dimensional shape contribute to the degree of retention.**

Superdex 75

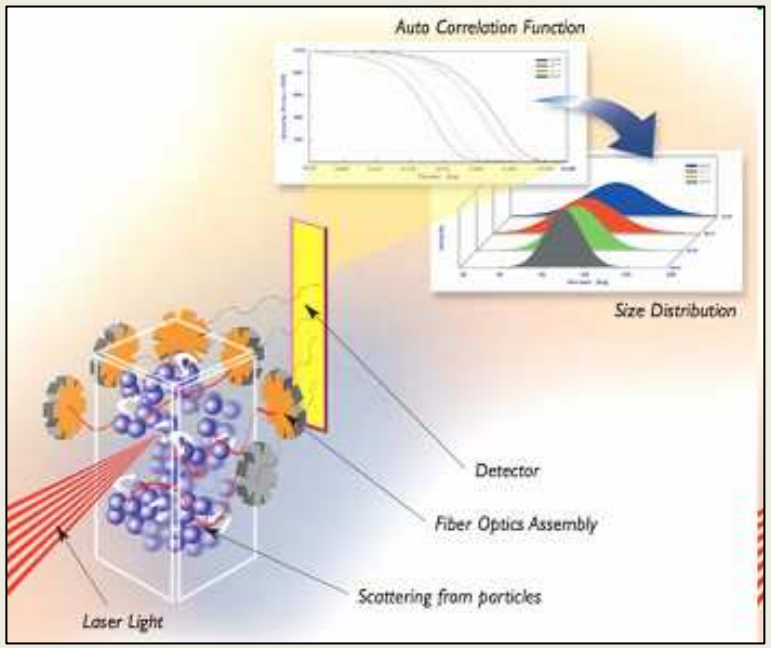


Elution volume 65 ml

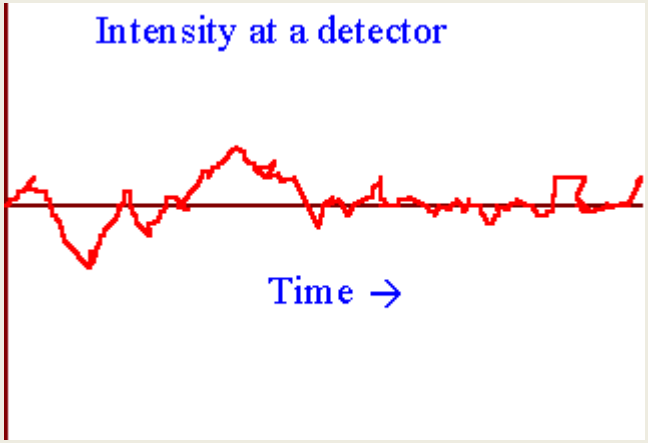


Elution volume 65 ml

Dynamic light scattering (DLS)



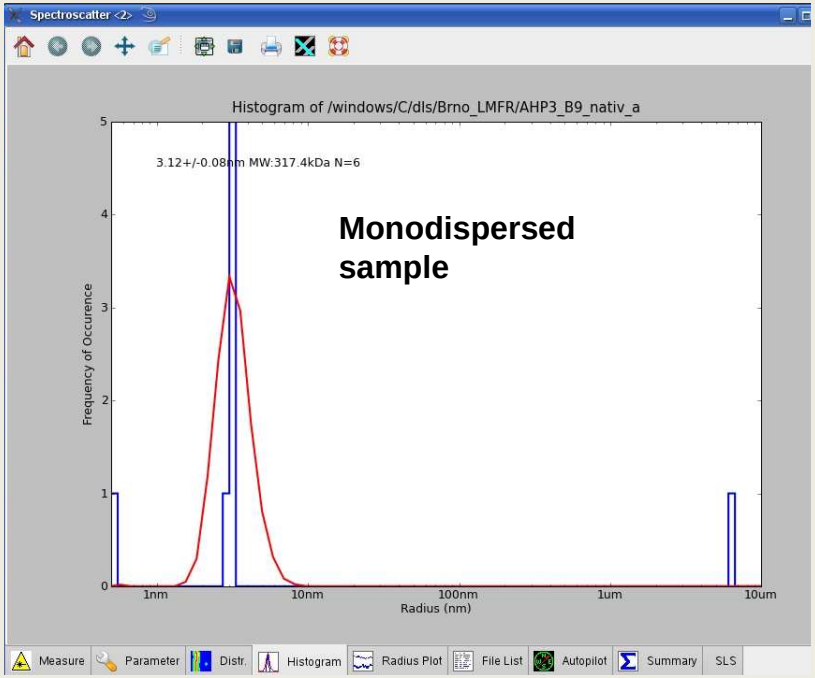
DLS is a method to determine hydrodynamic sizes, polydispersities and aggregation effects of protein samples. It can be understood as a method to measure the velocity of the Brownian motion.

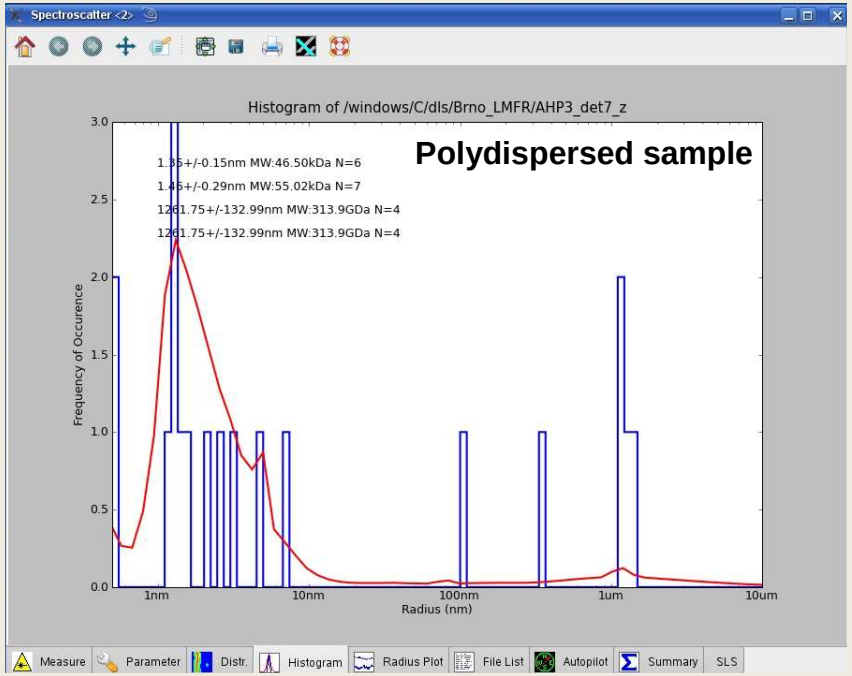


- The laser is focused in the sample cell and the particles scatter the light in all directions.
- Fluctuations in the scattering light intensity are measured as a function of time over a time scale of approximately 100 ns–30 ms.
- Those fluctuations are due to the Brownian motion of scattering particles.
- The time scale of the scattering fluctuations is directly related to the diffusion coefficient of the scattering particles, which in turn is related to protein size (Stokes-Einstein equation).
- In DLS, larger particles produce a very strong scattering intensity signal.

Dynamic light scattering (DLS)

Examples of results

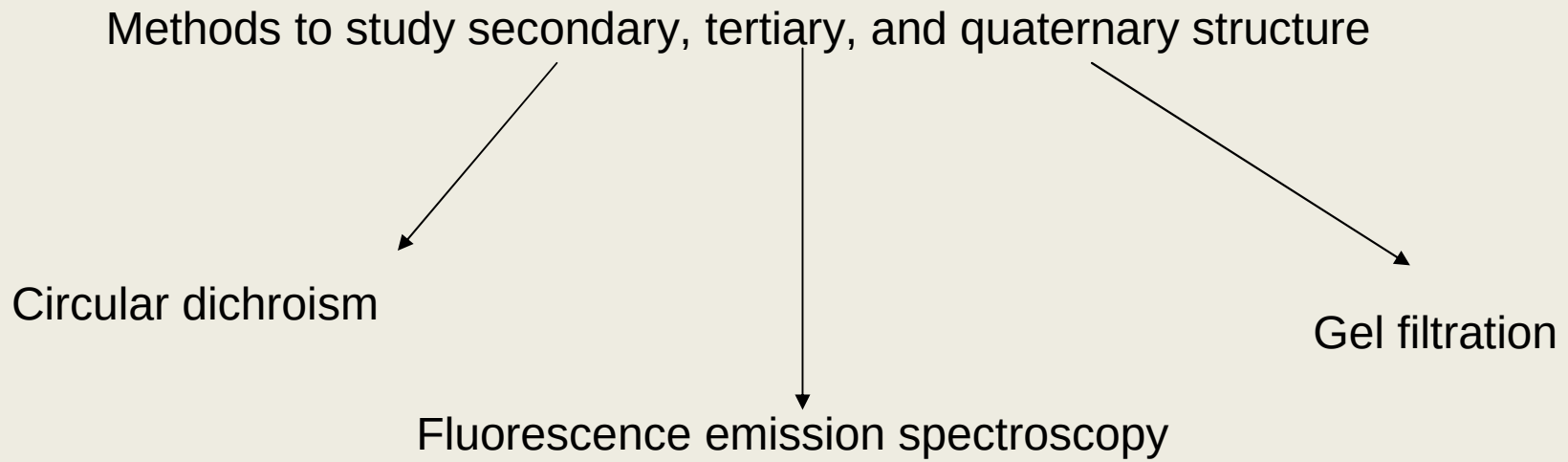




How to determine the protein quality parameters?

STUDY OF PROTEIN FOLDING INTO THE NATIVE STRUCTURE

The atomic resolution structure of a protein can only be revealed by the methods of X-ray crystallography and NMR.
- time consuming (not for rapid screening)
- not easy to obtain protein in high quality for these types of experiments



Fluorescence emission spectroscopy

Amino acids with intrinsic fluorescence properties: phenylalanine, tyrosine, tryptophan; but only tyrosine and tryptophan are used experimentally because their quantum yields (emitted photons/excited photons) are high enough to give a good fluorescence signal. So this technique is limited to proteins having either Trp or Tyr, or both.

At an excitation wavelength of 280 nm, both Trp and Tyr will become excited. To selectively excite Trp only, a 295 nm wavelength must be used.

Trp and Tyr residues can be used to follow protein folding because their fluorescence properties (quantum yields) are sensitive to their environment, which changes when a protein folds/unfolds. In the native folded state, Trp and Tyr are generally located within the core of the protein, whereas in a partially folded or unfolded state they become exposed to solvent.

1. In a hydrophobic environment (buried within the core of the protein), Tyr and Trp have a high quantum yield and therefore a high fluorescence intensity. In a hydrophilic environment (exposed to solvent), on the other hand, their quantum yields decrease, leading to low fluorescence intensity.

2. The wavelength of the emission maximum also reflects the hydrophobic (λ em 308 nm for azurin indicating that Trp is deeply buried within the core of the protein) and hydrophilic (λ em 352 nm for glucagon indicating that Trp is exposed to the solvent) environment.

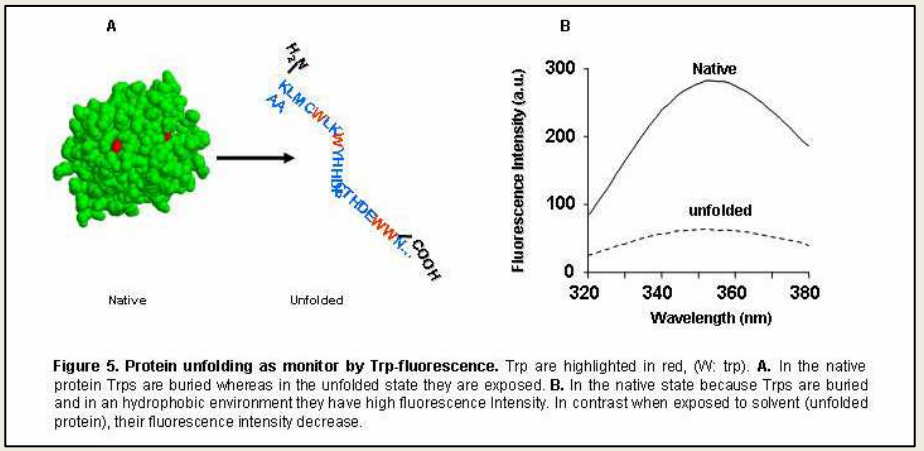


Figure 5. Protein unfolding as monitor by Trp-fluorescence. Trp are highlighted in red, (W: trp). **A.** In the native protein Trps are buried whereas in the unfolded state they are exposed. **B.** In the native state because Trps are buried and in an hydrophobic environment they have high fluorescence Intensity. In contrast when exposed to solvent (unfolded protein), their fluorescence intensity decrease.

Measurement of fluorescence intensity

The most common ways of unfolding a protein are using chaotropic agents (urea, quanidin chloride), changing the pH, or increasing temperature. It is possible to measure either steady state or kinetic unfolding.

For example, the protein is unfolded by increasing temperature, so at each temperature the protein undergoes unfolding and reaches an equilibrium state corresponding to a partially folded or fully unfolded state depending on the conditions. For each temperature, the fluorescence emission of Trp or Tyr is measured and compare to that native state protein.

- Fluorescence intensity (FI) will change upon unfolding. Following the change of this parameter, an unfolding curve is generated by plotting FI = f (temperature).

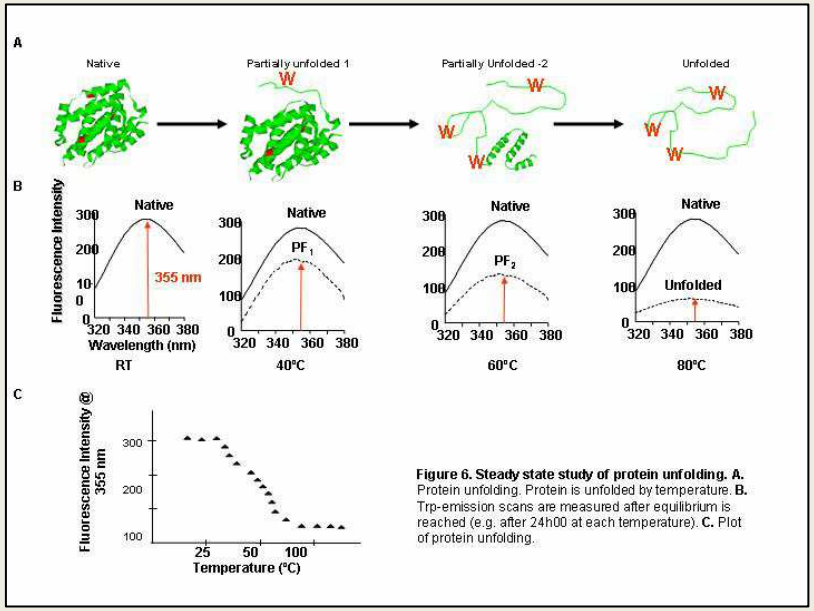
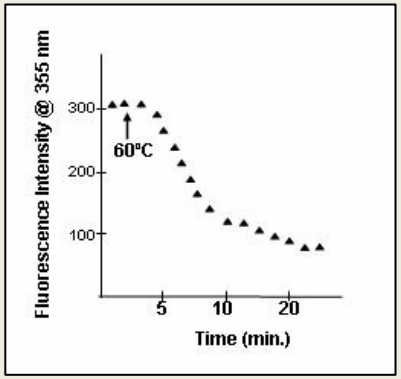


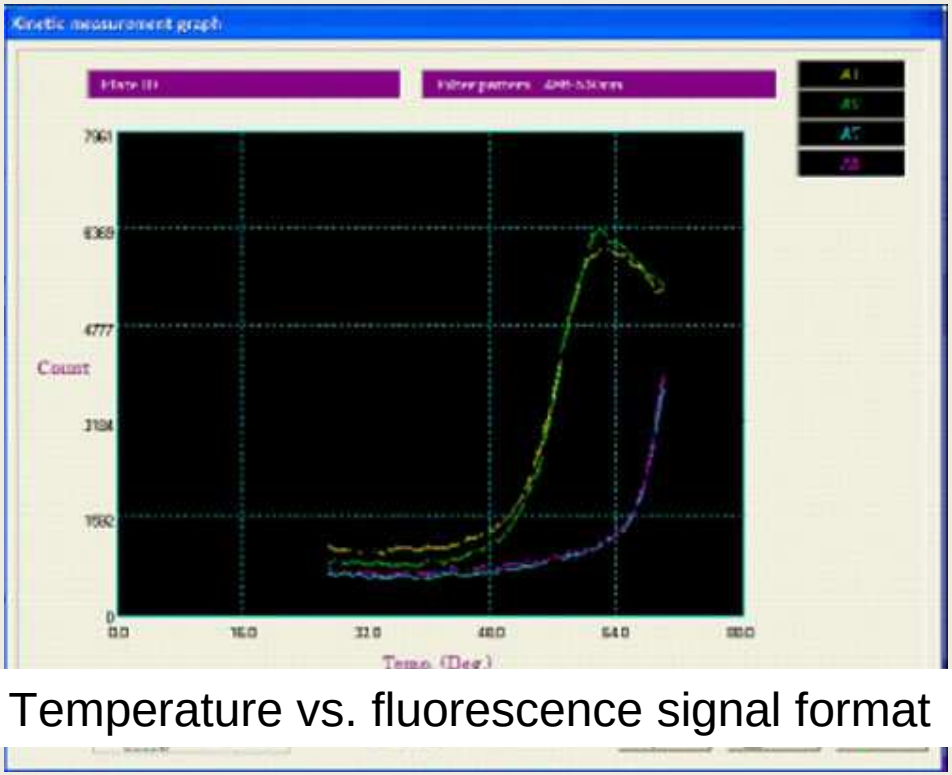
Figure 6. Steady state study of protein unfolding. A. Protein unfolding. Protein is unfolded by temperature. **B.** Trp-emission scans are measured after equilibrium is reached (e.g. after 24h00 at each temperature). **C.** Plot of protein unfolding.

- **For kinetic studies, the protein is kept at one temperature and its unfolding reaction is followed in time.**

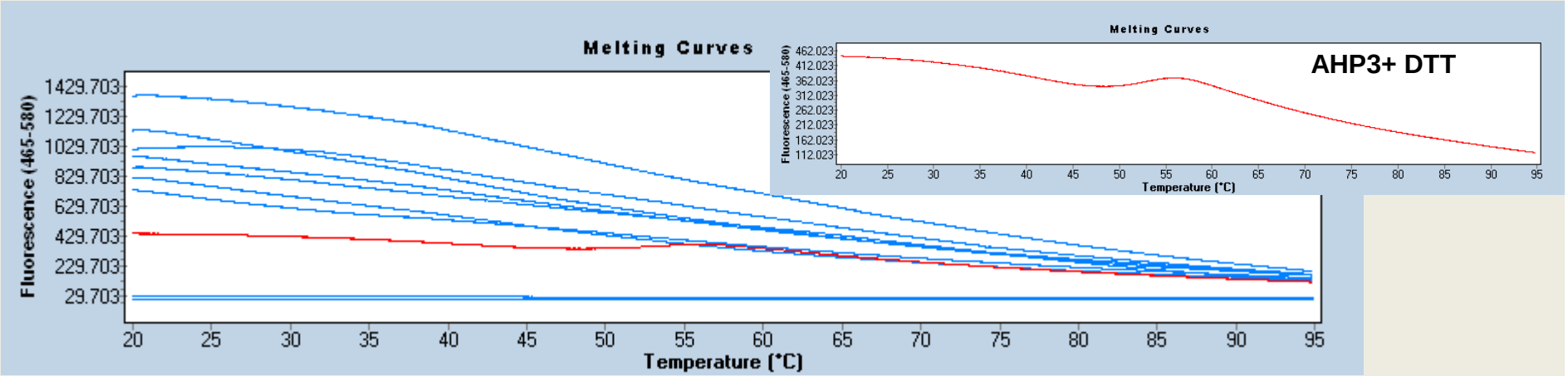


Measurement of fluorescence intensity

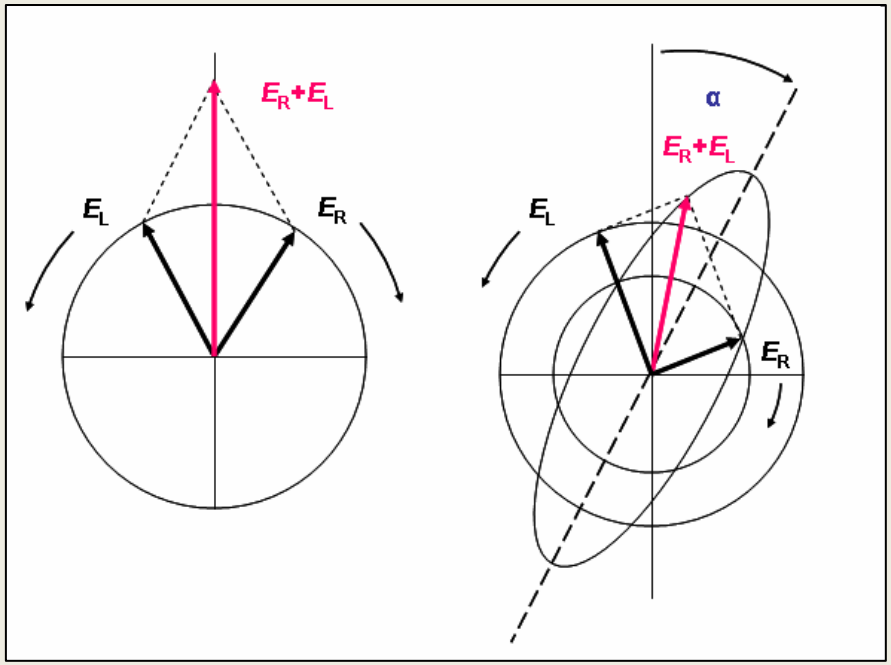
➢ As mentioned, the previous technique is limited to proteins containing Trp or Tyr residues. It is also possible to use probes that bind specifically to hydrophobic protein residues (Sypro Orange). Those surfaces are hidden in a native protein but exposed in partially folded or fully unfolded proteins.



Temperature vs. fluorescence signal format



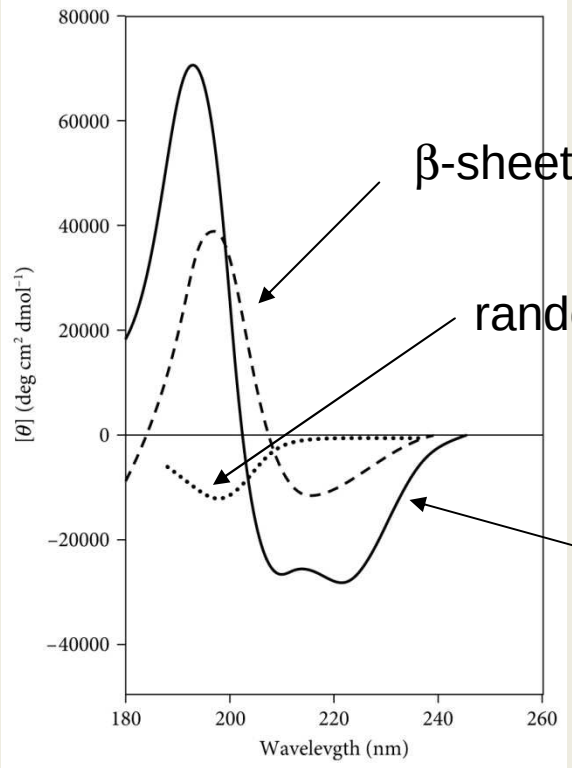
Circular dichroism spectroscopy – exploring the secondary structure of proteins



The technique depends on the difference in absorbance between left and right circularly polarized light beams. CD can be observed in the range of wavelengths at which chiral molecules absorb light.

The relevant spectral region is in far UV, i.e. from 240 nm down to 180 nm, where peptide bonds absorb light. In this region, the different types of regular secondary structure, such as alpha-helix and beta-sheet, exhibit a characteristic spectral pattern.

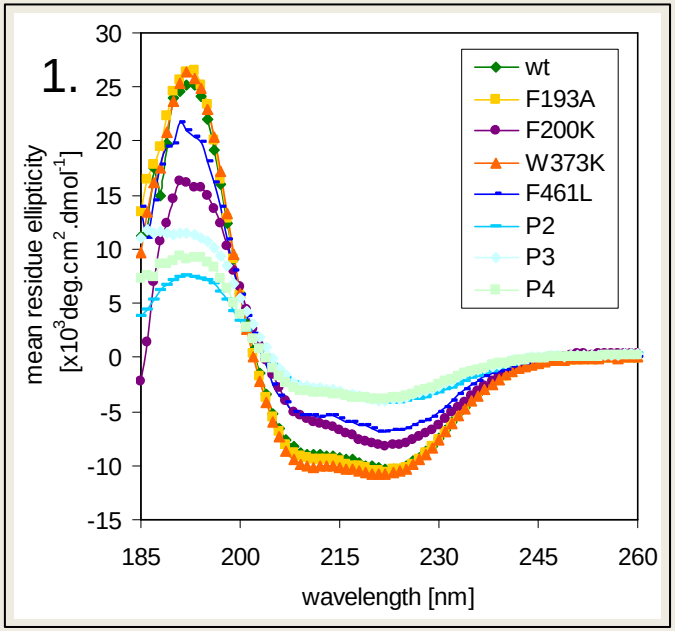
Circular dichroism spectroscopy – exploring the secondary structure of proteins



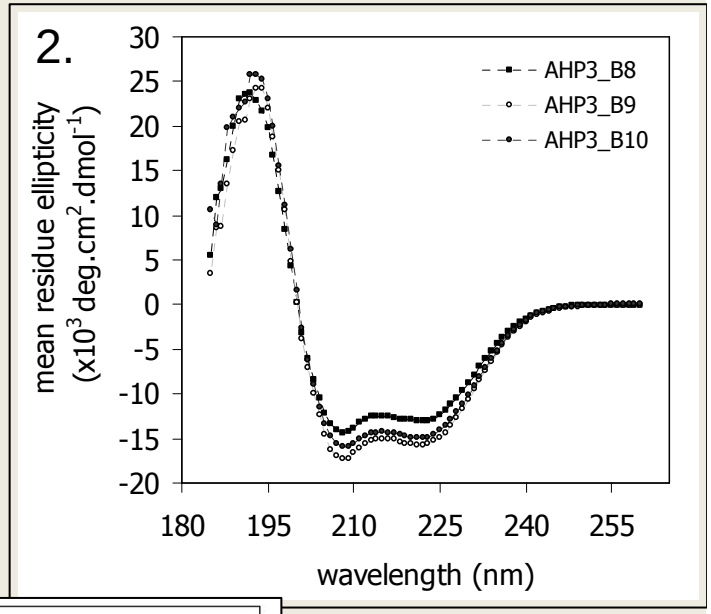
α-helix spectrum shows the characteristic two negative minima at 208 and 222 nm and a positive maximum at 193 nm. β–sheet spectrum shows a single negative minimum at about 215 nm and a positive maximum at 196 nm (both of these are much smaller than the signal for α-helix). The random coil shows only a very small signal above 210 nm and a small negative minimum at about 198 nm.

Circular dichroism spectroscopy – exploring the secondary structure of proteins

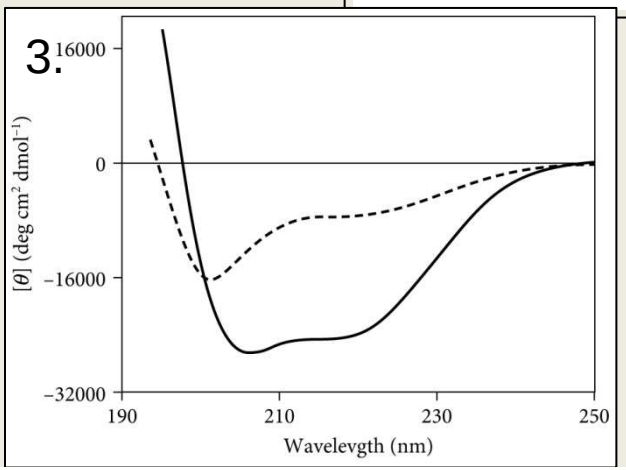
1. Maize β-glucosidase, structure (b/a) 8 barrel
Far-UV circular dichroism spectra of wild type b-glucosidase enzyme and its mutants F193A, F200K, W373K, F461L, P2, P3 and P4



2. AHP proteins, structure 6 a-helix bundle
Far-UV circular dichroism spectra of three purification fractions of *Arabidopsis thaliana* histidine phosphotransfer protein AHP3 eluted at different salt concentrations (B8 – 240 mM NaCl, B9 – 254 mM NaCl, B10 – 260 mM NaCl)



3. Far-UV spectra of CGRP (calcitonin gene related peptide) in phosphate buffer of pH 7 (dashed line) and in the same buffer + 50% trifluoroethanol (solid line)



Determination of quaternary structure and activity of β-glucosidase and its mutant forms

Gel filtration chromatography may be used to analyze the molecular size of macromolecules.

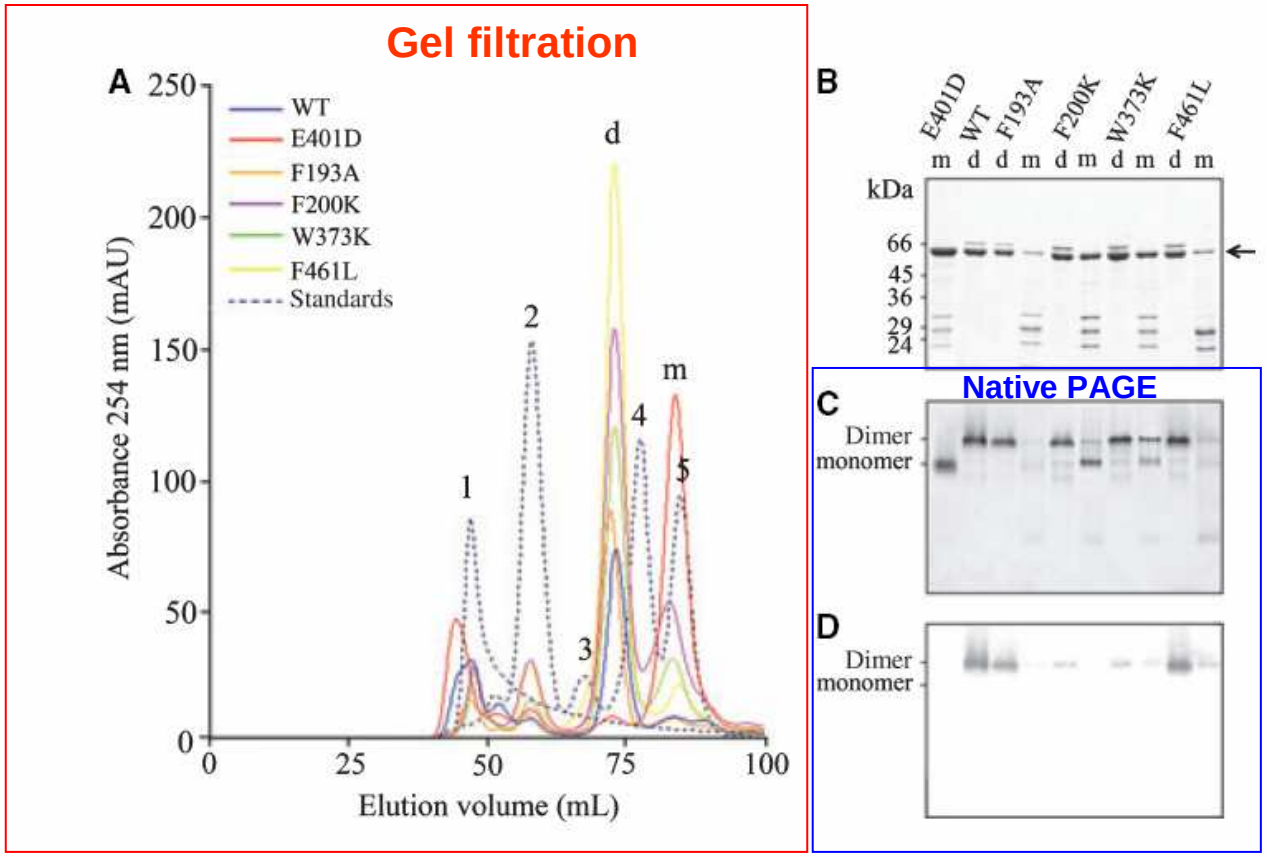
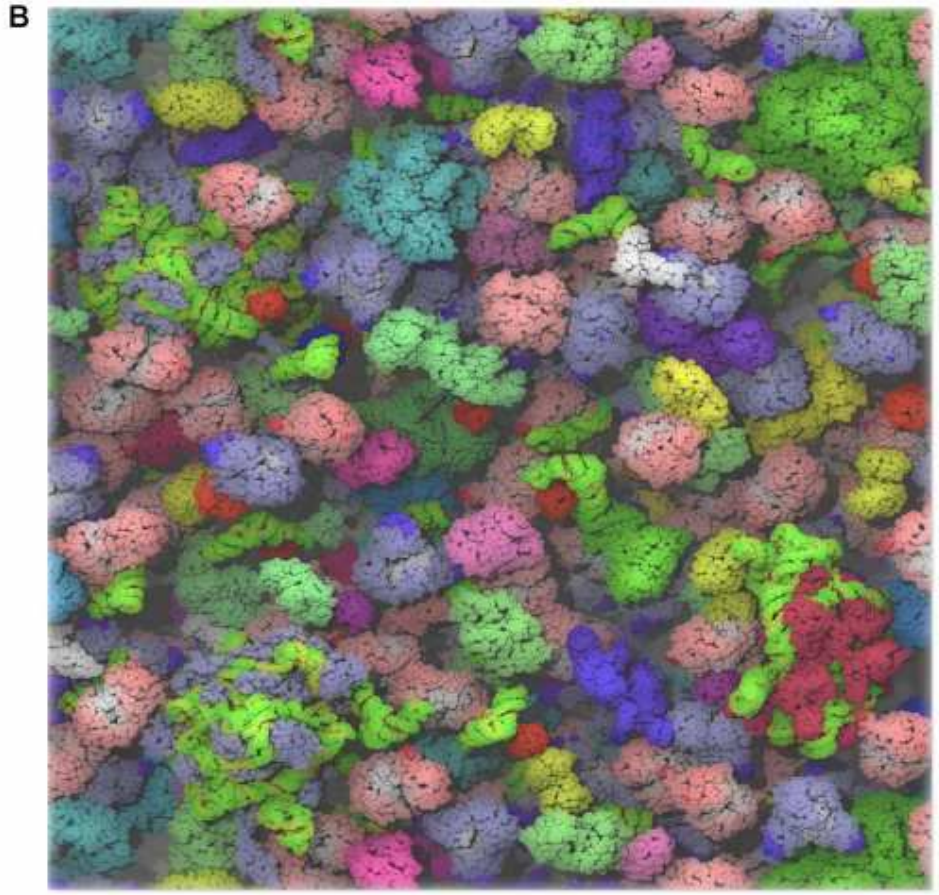


Fig. 5. Quaternary structure of wild-type and mutant Zm-p60.1 β-glucosidases. (A) Elution profiles of wild-type and mutant Zm-p60.1 β-glucosidases from the HighLoad 16/60 Superdex 200 column. A sample (1.5 mL) of each enzyme purified by metal chelate affinity chromatography was applied to the column and eluted with elution buffer (50 mM Tris/HCl, 500 mM NaCl; pH 7.00). Fractions corresponding to peaks d and m were collected and analyzed by (B) Coomassie Brilliant Blue-stained SDS/PAGE, (C) Coomassie Brilliant Blue-stained native-PAGE and (D) in-gel activity staining of native-PAGE gels. Peaks 1, 2, 3, 4 and 5 correspond to Blue Dextran 2000, ferritin (M_r 440 kDa), aldolase (M_r 158 kDa), BSA (M_r 67 kDa) and ovalbumin (M_r 43 kDa), respectively, used as standards. Arrow marks positions of the wild-type and mutant Zm-p60.1 polypeptides in SDS/PAGE.

PROTEIN STABILITY



First structurally detailed molecular model of bacterial cytoplasm (combination of proteomic data with high resolution structural data)

Name	Mw	#
Adk	24	14
AhpC	187	7
Asd	80	4
Bcp	11	8
CspC	7	72
CysK	64	13
DapA	125	2
DnaK	41	11
Efp	20	14
Eno	91	18
Fba	78	6
Frr	21	7
FusA	69	22

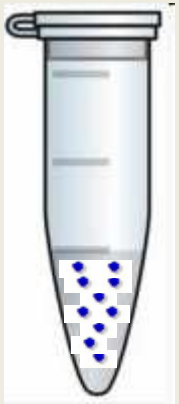
Name	Mw	#
GapA	142	10
GlnA	621	1
GltD	94	3
GlyA	91	15
GpmA	55	4
Hns	5	7
Hup	15	12
IcdA	92	43
IlvC	54	18
Mdh	65	13
MetE	84	213
Mop	845	2

Name	Mw	#
PanB	140	2
Pgk	41	26
Pnp	190	3
Ppa	116	9
PpiB	18	7
PurA	94	4
PurC	42	7
Pyr	308	3
RpiA	46	3
Rpo	260	4
SerC	79	11
SodA	46	13
SodB	42	9

Name	Mw	#
Suc	142	4
Tig	48	9
TpiA	54	5
Tsf	61	12
TufA	84	181
Upp	45	11
UspA	31	7
50S	1,355	10
30S	788	10
tRNA-C	24	37
tRNA-Q	24	37
tRNA-F	25	37
GFP	26	8

Figure 1. The cytoplasm model. A. Schematic inventory of the contents of the cytoplasm model. **B.** Rendering of the cytoplasm model at the end of a Brownian dynamics simulation performed with the 'full' energy model (see text). RNA is shown as green and yellow. This figure was prepared with VMD [110].
doi:10.1371/journal.pcbi.1000694.g001

Proteins removed from their natural cellular environment are subjected to a variety of external factors that may lead to a loss (or impairment) of their biological activity as a result of both covalent and non-covalent structural alteration.



PROTEIN STABILITY

The shelf life of a protein depends on the intrinsic nature of the protein and on storage conditions.

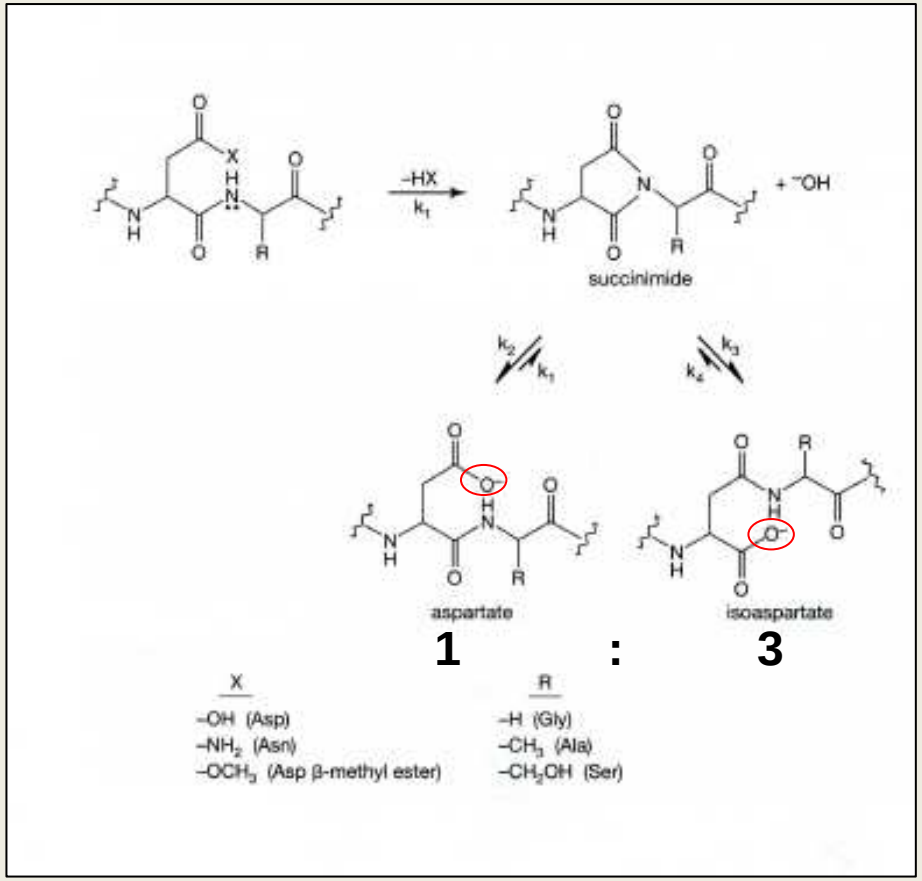
Many factors cause protein inactivation during purification and storage:

- Nearly every property of a protein, except for its primary amino acid sequence, can be shown to vary with the solution conditions, such as pH, temperature and ion concentration.
- Loss of an essential cofactor.
- Exposure to proteases, oxygen, or heavy metals.
- Changes in physical conditions such as freezing and thawing.

CHEMICAL REACTIONS AFFECT PROTEIN STABILITY

Succinimide (cyclic imide) formation following deamination

Formation of β-isoaspartate as a result of deamination of asparagyl residues or isomerization of aspartyl residues is a major source of instability in proteins and peptides, especially **at neutral and alkaline pH after prolonged storage**.



For instance, the disposition of an asparagine residue followed by a small hydrophilic amino acid residue such as glycine, serine or threonine in a polypeptide chain can lead to deamination of an asparagine and formation of α-aspartic acid and/or β-isoaspartate via an intramolecular rearrangement that produce a succinimide (cyclic imide, unstable under physiological conditions) intermediate. This phenomenon occurs at a rapid rate and conveys extra negative charge to the protein. β-isoaspartate-bearing protein are reported to be specific substrates for a widely distributed methyltransferase enzyme that uses S-adenosyl-L methionine as its methyl donor. It has been proposed that this class of methyltransferases specifically recognizes proteins with incurred chemical changes (β-isoaspartate formation) and that the methylation reaction may be the first step in protein degradation.

CHEMICAL REACTIONS AFFECT PROTEIN STABILITY

Aspartyl-prolyl bond cleavage

Specific cleavage at Asp-Pro peptide bonds in a polypeptide chain can occur by exposure to acid conditions (e.g. 10% acetic acid with pH 2.5, 70–75% formic acid) at moderate temperatures (37℃, 40℃) for periods u p to 120 hours.

Pyroglutamate formation

Cyclization of amino terminal glutamine residues to pyroglutamyl residues under mild acidic conditions is a frequent cause of protein modification.

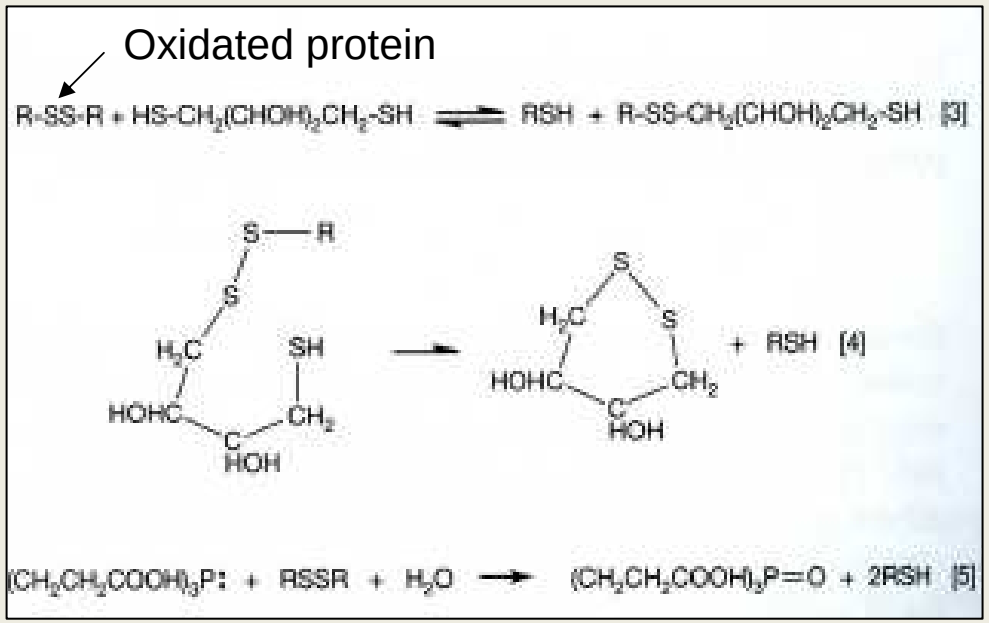
CHEMICAL REACTIONS AFFECT PROTEIN STABILITY

Oxidation

Oxidation of proteins **by molecular oxygen** during isolation and storage can lead to their inactivation and/or aggregation.

Cysteine is particularly prone to oxidation, as are methionine and tryptophan residues.

Reducing agents that are primarily used to protect free SH-groups from oxidation (particularly of cysteine and methionine residues): dithiothreitol (DTT) or 2-mercaptoethanol (2-ME), *tris*(2-carboxyethyl)phosphine (TCEP).



→ 2-ME

→ DTT

→ TCEP

CHEMICAL REACTIONS AFFECT PROTEIN STABILITY

Oxidation

It is advisable to add the metal chelating agent EDTA (or EGTA) to protein solution, as well as a reducing sulfhydryl reagent, for complex trace metals such as Cu, Fe, and Zn. These trace metals can either bind to sulfhydryl (cysteine) residues directly or catalyze their oxidation by molecular oxygen.

- DTT is preferred to 2-mercaptoethanol, because DTT reduces disulfides quantitatively (does not form mixed disulfides).
- The higher the pH and temperature, the shorter the half-lives of the thiol reagents.

TABLE A3.4. Half-life of thiol compounds in solution

Conditions	Half-life (hours)			
	2-Mercaptoethanol	DTT	Glutathione	3-Mercaptopropionate
pH 6.5, 20°C	>100	40	16	7
pH 7.5, 20°C	10	10	9	5
pH 8.5, 20°C	4.0	1.4	1.3	4.5
pH 8.5, 0°C	21	11	8	13
pH 8.5, 40°C	1.0	0.2	0.2	1.6
pH 8.5, 20°C + 0.1 mM Cu^{2+}	0.6	0.6	1.2	0.4
pH 8.5, 20°C + 1.0 mM EDTA	>100	4	70	>100

Reproduced, with permission of Wiley, from Stevens et al. (1983 [© International Union of Biochemistry & Molecular Biology]). All thiol compounds were dissolved in 0.1 M potassium phosphate buffer.

DTT is stable 1 week at 2-8℃, pH 7,75, protected from atm ospheric oxygen.

- TCEP is a more stable, faster and stronger reductant than DTT at pH values below 8.0. It resists air oxidation and is stable over a wide pH range (1.5–8.5).
- TCEP is recommended for long-term storage of proteins in the absence of metal chelators (EDTA, EGTA), because Fe3+ and Ni2+ catalyzes DTT oxidation.

PROTEOLYSIS DURING PURIFICATION AND STORAGE

A simple test for proteolytic degradation is to incubate the sample extract or partially purified target protein at 30–37℃, withd rawing aliquats at various time points and assaying them for biological activity. Caution must be exercised with this method of proteolysis detection, as the target protein may be partially degradated, yet retain full biological activity. Therefore, protein size and microheterogeneity have to be monitored by SDS-PAGE and following western blotting and/or by mass spectrometry. If loss of activity occurs upon prolonged incubation at moderate temperature (e.g. 30℃), yet the size of the protein remains unaltere d, the structural integrity (e.g. folding) should be investigated using fluorescence, circular dichroism, etc.

PROTEOLYSIS DURING PURIFICATION AND STORAGE

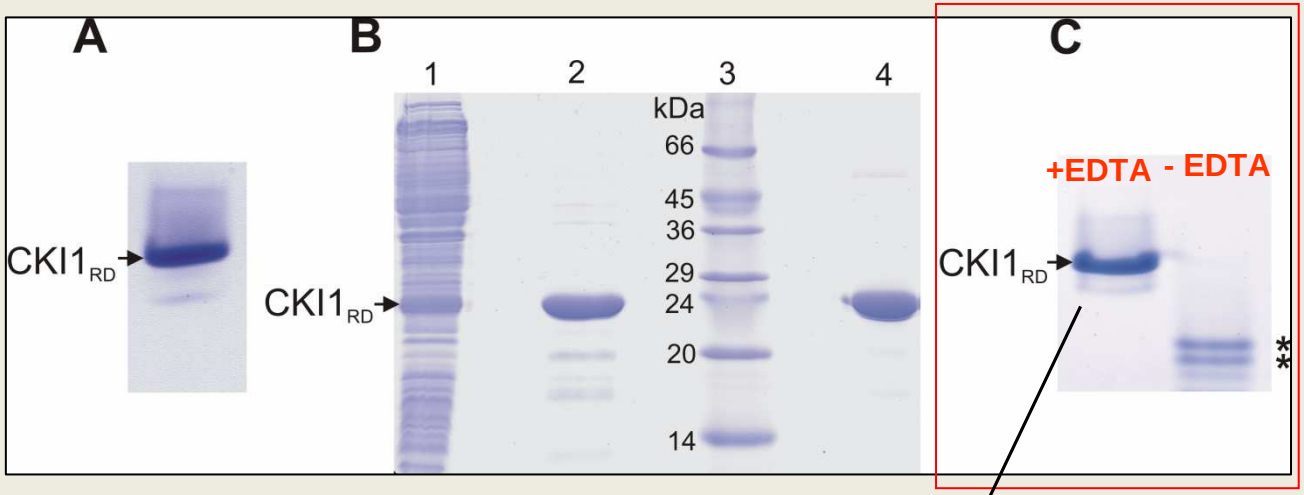
If loss of biological activity indicates that proteolysis is a problem, then protease inhibitors must be added to the sample and during all future attempts at purification of that target protein.

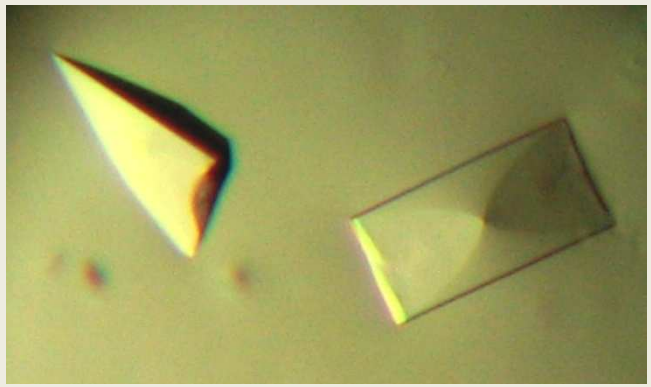
Table 2. Common Protease Inhibitors

Protease Inhibitor	Target Protease	Working Concentration
PMSF (Phenylmethylsulfonyl fluoride)	Serine proteases	0.1-1 mM
Benzamidine	Serine proteases	1 mM
Pepstatin A	Acid proteases	1 µg/ml
Leupeptin	Thiol proteases	1 µg/ml
Aprotinin	Serine proteases	5 µg/ml
Antipain	Thiol proteases	1 µg/ml
EDTA and EGTA	Metalloproteases	0.1-1 mM

PROTEOLYSIS DURING PURIFICATION AND STORAGE

C: Stability of the purified $CKI1_{RD}$ after three months storage at 4℃





Protein was co-purified with traces of metalloproteases.

PROTEIN CONCENTRATION AFFECTS STABILITY

- In general, proteins are **less stable** at low concentrations (**<50 μg/ml**). Under these conditions, multiple subunit proteins and cofactors tend to dissociate and physical losses due to adsorption to surfaces can become significant.
- When total protein concentration falls below ~**50 μg/ml**, protein loss can occur due to strong and irreversible adsorption of the protein to a variety of surfaces, including glass, plastic, and various types of filtration media used for concentrating, clarifying, or sterilizing proteins.
- It is **advisable** to keep protein concentration as high as possible (**e.g. >1 mg/ml**) during purification and storage. This is relatively easy to achieve during the early stages of a purification procedure; however, it becomes more difficult during the later stages of purification or in the case of purification of low abundant proteins isolated from natural sources. In these circumstances, it is imperative that **solvent additives**, such as polyethylene glycol (0.05% v/v) and nonionic detergents (0.02% w/v, Triton-X-100, or Tween-20), be included in all buffers and eluents used in the purification procedure.
- Protein solutions lower than 1 mg/ml should be concentrated as rapidly as possible using methods such as dialysis and lyophilization.
- In situations where rapid concentration of a dilute protein solution is not possible and the presence of an exogenous protein can be tolerated, the addition of ~1 mg/ml bovine serum albumin (BSA) has been shown to be very effective.
- On the other hand, **protein aggregation is generally concentration dependent**.

Protein aggregation – a major event of physical instability of proteins

- Under certain conditions (or simply with time), the secondary, tertiary and quaternary structure of a protein may change and lead to protein unfolding and/or aggregation (the assembly of individual protein molecules into amorphous, multimeric states).
- Protein aggregates may have reduced or no activity, solubility, and altered immunogenicity.

Mechanism of protein aggregation

- Proteins aggregate to minimize thermodynamically unfavorable interactions between a solvent and exposed hydrophobic protein residues.
- Hydrophobic interaction, i.e. the reluctance of non-polar groups to be exposed to water, is considered to be a major driving force for both protein folding and aggregation. Both protein aggregation and folding represent a balance of exposed and buried hydrophobic surface areas. The balance is so delicate that a change of one amino acid in a protein may substantially change its aggregation behavior.
- Other possible mechanisms: formation of non-native disulfide bonds, electrostatic interactions…..
- Protein aggregation may be induced by a variety of physical factors, such as temperature, ionic strength, foaming, protein concentration, pH shift, vortexing, etc. These factors can increase the hydrophobic surface of proteins, causing aggregation.

STABILIZATION IS NECESSARY FOR LONG-TERM STORAGE OF PROTEINS

Solvent additives that stabilize the protein:

Osmolyte stabilizers

= a class of weakly charged compounds with low molecular weight that are used as co-solvents for stabilizing purified proteins for storage and during freezing, thawing and lyophilization. They enhance stability through a preferential hydration mechanism as well as a solvophobic effect (TMAO).

- Osmolyte stabilizers include sugars (glycerol, xylitol, PEG), amino compounds and small neutral amino acids (glycine, alanine), including their derivatives, as well as large dipolar molecules such as trimethylamine N-oxide (TMAO).

TABLE A3.5. Solvent additives that stabilize or destabilize proteins in solution

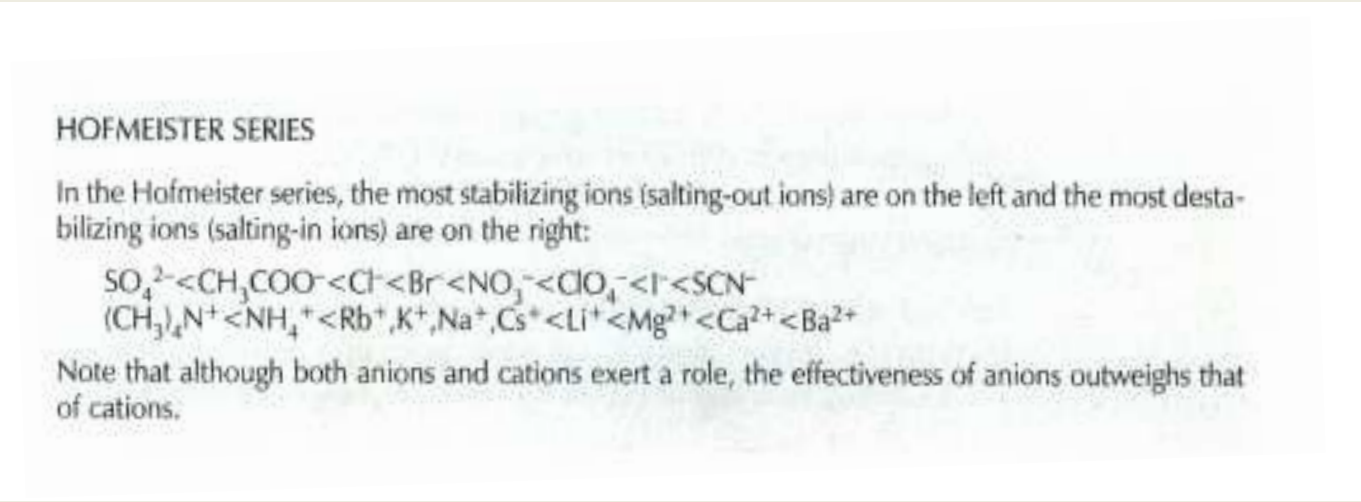
Compounds	Mode of Action	Working Concentration
Osmolytic stabilizers[a]		
Polyols and sugars glycerol, erythritol, arabitol, sorbitol, mannitol, xylitol, mannisidomannitol, glucosylglycerol, glucose, fructose, sucrose, trehalose, isofluoroside	These stabilize the lattice structure of the water, thereby increasing surface tension and viscosity. In addition, they stabilize hydration shells and protect against aggregation by increasing the molecular density of the solution without changing the dielectric constant.	10–40%
Polymers dextrans, levans, polyethylene glycol	Polymers increase molecular density and solvent viscosity, thus lowering protein aggregation in a single-phase system. At high polymer concentration, a two-phase system develops and the protein aggregates in the phase in which its concentration is highest.	1–15%
Amino acids and their derivatives glycine, alanine, proline, taurine, betaine, octopine, glutamate, sarcosine, γ-amino-butyric acid, trimethylamine *N*-oxide (TMAO)	Small amino acids with no net charge, such as glycine and alanine, have weak electrostatic interactions with proteins. Octopine is a derivative of arginine that is less denaturing to proteins. TMAO stabilizes proteins even in the presence of denaturants such as urea. Most of these compounds increase the surface tension of water.	20–500 mM

STABILIZATION IS NECESSARY FOR LONG-TERM STORAGE OF PROTEINS

Solvent additives that stabilize the protein:

Ionic stabilizers

- Certain ionic compounds and neutral salts can stabilize proteins in solution.
- The stabilizing effect of neutral salts varies with the position of the constituent ions in the Hofmeister lyotropic series, which reflects ionic effects on protein solubility, association–dissociation equilibria, and enzyme activity. The Hofmeister series ranks both cations and anions according to their stabilizing effects.



HOFMEISTER SERIES

In the Hofmeister series, the most stabilizing ions (salting-out ions) are on the left and the most destabilizing ions (salting-in ions) are on the right:

$SO_4^{2-} < CH_3COO^- < Cl^- < Br^- < NO_3^- < ClO_4^- < I^- < SCN^-$

$(CH_3)_4N^+ < NH_4^+ < Rb^+, K^+, Na^+, Cs^+ < Li^+ < Mg^{2+} < Ca^{2+} < Ba^{2+}$

Note that although both anions and cations exert a role, the effectiveness of anions outweighs that of cations.

STABILIZATION IS NECESSARY FOR LONG-TERM STORAGE OF PROTEINS

Ionic stabilizers

➢ In general, stabilizing ions favor the ordered or folded (native) conformation of a protein in aqueous solution by strengthening intramolecular contacts. This helps to prevent the unfolding process, the initial event in protein aggregation/inactivation. Usually, salts that destabilize the form of a protein increase its solubility in water of the hydrophobic amino acid side chains that normally exist in the “interior” of an ordered (native) protein structure.

Ionic stabilizers[b]		
Salts		
citrate, sulfates, acetate, phosphates, quaternary amines	Larger anions shield charges and can stabilize proteins at low concentrations. At high concentrations, they lead to precipitation due to competition for water molecules.	20–400 mM
Ionic destabilizers		
Salts		
chlorides, nitrates, thiocyanates	Although generally less stabilizing than large ions (especially polyvalent ions), these are useful for charge shielding at lower concentrations.	20–400 mM
Denaturants (chaotrophs)		
urea, guanidinium salts, trichloroacetates, cetylmethyl-ammonium salts, organic solvents	Denaturants either stabilize the unfolded state of proteins (urea) or perturb protein structure by interfering with hydrogen bonding or disturbing the hydration shell.	0.2–8 M

Adapted, in part, with permission, from Schein (1990).
[a]In general, the osmolytic stabilizers have little direct interaction with proteins, but affect the bulk solution properties in water.
[b]These affect enzyme reactions. Their stabilizing effects on proteins occur within a concentration range much narrower than that of osmolyte stabilizers

➢Initial use of a weak ionic stabilizer, such as guanidinium acetate (to arrest protein unfolding), followed by slowly changing to an intermediate stabilizer (e.g. guanidinium sulfate) and finally to a strong ionic stabilizer (e.g. sodium sulfate) allows for a protein with intermediate folded forms to return gradually to a more native-like structure.

STABILIZATION IS NECESSARY FOR LONG-TERM STORAGE OF PROTEINS

Low temperature storage

- Although chemical modification and/or proteolytic degradation of proteins can occur at moderate temperatures, the extent of these changes is impaired at increased temperatures.
- High number of commercially available biochemicals that are supplied in >3M ammonium sulfate or ~50% glycerol. Typically, proteins stabilized in this manner should be stored at refrigerator temperatures (4–6°C), although more labile proteins should be stored at -20℃ and extremely labile prot eins at even lower temperatures (-70℃).
- It is important to determine the proper storage conditions for the target protein because certain proteins (so-called “cold labile” proteins) are more stable at ambient temperature than in a refrigerator. Cold denaturation of proteins (i.e. unfolding of proteins at low temperatures) has been well characterized and has been shown to be an inherent property of the protein itself (and distinct from “freezing inactivation”).

STABILIZATION IS NECESSARY FOR LONG-TERM STORAGE OF PROTEINS

Comparison of protein storage conditions

Storage conditions				
Characteristics	Solution at 4℃	Solution in 25–50% glycerol or ethylene glycol at -20℃	Frozen at -20℃ to -80℃ or in liquid nitrogen	Lyophilized (usually also frozen)
Typical shelf life	1 month	1 year	Years	Years
Requires sterile conditions or addition of antibacterial agent	Yes	Usually	No	No
Number of times a sample may be removed for use	Many	Many	Once; repeated freeze-thaw cycles generally degrade proteins.	Once; it is impractical to lyophilize a sample multiple times.

STABILIZATION IS NECESSARY FOR LONG-TERM STORAGE OF PROTEINS

General considerations for protein storage

- Store the protein in a concentration of at least 1 mg/ml.
- If the concentration is lower, stabilize the protein by adding another protein, e.g. BSA, or certain additives.

Many compounds may be added to protein solutions to lengthen shelf life:

- Cryoprotectants such as glycerol or ethylene glycol added to a final concentration of 25–50% help to stabilize proteins by preventing the formation of ice crystals at -20℃ that destroy protein structure.
- Protease inhibitors prevent proteolytic cleavage of proteins.
- Anti-microbial agents such as sodium azide (NaN_3) at a final concentration of 0.02–0.05% (w/v) inhibit microbial growth.
- Metal chelators such as EDTA at a final concentration of 1–5 mM prevent metal-induced oxidation of SH groups and help to maintain the protein in reduced state.
- Reducing agents such as dithiothreitol (DTT) and 2-mercaptoethanol (2-ME) at a final concentration of 1–5 mM also help to maintain the protein in the reduced state by preventing oxidation of cysteines.